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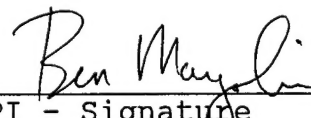
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## INTRODUCTION

Our project goal is to determine a function for the Grb7 protein. Grb7 is an SH2 domain protein that is overexpressed and bound to the receptor tyrosine kinase HER2 in a subset of breast cancers. In our original proposal and in last years progress report we discussed the role of HER2 in breast cancer. To briefly summarize HER2 is amplified and overexpressed in breast cancer and portends a poor prognosis for those patients with lymph node metastases (1). Upon activation, the HER2 tyrosine kinase phosphorylates itself as well as substrate proteins on tyrosine residues. Autophosphorylation of the receptor allows the receptor to bind a variety of SH2 domain proteins involved in signal transduction (2). The proteins that bind to autophosphorylated receptors usually contain Src Homology 2 (SH2) domains. Grb7 is one of the Grb family of SH2 domain proteins that was isolated by our group after screening bacterial expression libraries with the autophosphorylated Epidermal Growth Factor Receptor (3). It maps close to HER2 on human chromosome 17 and our group determined that it was coamplified and overexpressed with HER2 in breast cancer cell lines and primary breast cancers (4). Grb7 is closely related to two other SH2 domain protein called Grb10 and Grb14. Grb10, which was also originally isolated by our group, has been found by several groups to bind with high affinity to members of the insulin receptor family (5-7). However like Grb7 its function in tyrosine kinase signaling is unknown. Grb14 was recently isolated from a breast cancer cell line and may signal downstream of Platelet Derived growth Factor Receptor (8). Based on analysis of the Expressed Sequence Tagged (EST) database, these three protein appear to be the only members of this gene family. However, there may be many spliced forms of these proteins. All these proteins have SH2 domains and a conserved central domain of 300 amino acids that we have termed the GM domain because it is found in *Grbs* and the *C. elegans* gene, *Mig-10* (9). These proteins also share a conserved proline rich region in their amino-terminus (8). The *Mig-10* gene is required for embryonic migration of a sub population of neuronal cells (10). However unlike Grb7 and Grb10, *Mig-10* does not have an SH2 domain. The central region of the GM domain contains a pleckstrin homology (PH) domain (9). In the last few years accumulated evidence has suggested that PH domains are involved in binding polar head groups of phospholipids (11,12). The GM domain also contains a very conserved 100 amino acid region just amino-terminal to the PH domain. We know that the SH2 domain allows Grb7 to interact with HER2 and other tyrosine phosphorylated proteins (4). We have hypothesized that the GM domain is also involved in protein/protein interaction and that this interaction is crucial for Grb7 and Grb10 signaling. The goal of our research is determine the role of Grb7 in receptor tyrosine kinase signal transduction in normal and pathologic states.

Our initial approach to this problem has been two fold. First we overexpressed Grb7 in the MCF-7 breast cancer cell line that does not contain appreciable amounts of Grb7. In these cells we could detect no effects of Grb7 on EGF or HER2 mediated growth of cells or cell migration in culture. We felt that we may not have seen an effect because these cells were already transformed and

planned to express Grb7 in nontransformed epithelial cells. Our second major strategy has been to search for Grb7 binding partners, especially proteins that interact with the GM domain. Initial attempts to study this question using bacterial expression cloning was unsuccessful. Accordingly we have switched to examining proteins from epithelial cells that interact with the GM domain or full length Grb7. Our research over the last year has continued to focus on these issues.

## BODY

Several studies have been undertaken in the past year. Our goals as outlined last year were 1) expression of Grb7 in the nontransformed MCF-10A breast cancer cell line and subsequent studies on the effect of Grb7 overexpression on cell growth and migration. 2) Identification of Grb7 binding proteins using fusion proteins and coimmunoprecipitation. In a further attempt to understand Grb7 function we have begun a new project examining the role of Grb7 in mouse development.

**1) Grb7 expression in normal epithelial cells.** Our initial plans for this year's research was to express Grb7 in the nontransformed breast cancer cell line, MCF-10A. We have found little or no endogenous Grb7 expression in these cells. Unfortunately we were unable to obtain MCF-10A cell lines that stably overexpressed Grb7. This was despite using the same techniques we had used to transfect MCF-7 cells (last year's report). However we still felt it was important to overexpress Grb7 in normal epithelial cells and turned to the MDCK cell line. MDCK is a cell line from the dog renal epithelium that normally expresses Grb7 (collaboration with Dr. Morag Park-McGill University). These cells represent a good experimental system because they form branching epithelial structures in collagen gels in response to Hepatocyte Growth Factor (HGF). Breast epithelial cells display a similar response to HGF and both MDCK and mammary epithelial cells migrate (scatter) in response to HGF (13,14). Dr. Park's group in collaboration with our laboratory has found that Grb7 is able to bind to the HGF Receptor and thus Grb7 might be necessary for epithelial cell responsiveness to HGF. This is intriguing because of the relationship of Grb7 to Mig-10, a gene necessary for cell migration in *C. Elegans* (10). Thus we felt overexpression of Grb7 in MDCK cells would allow us to further assess the role of Grb7 in growth factor mediated growth, tubulogenesis and migration.

We used two different Grb7 constructs for the transfections of MDCK cells. The first was Grb7 with a myc tag at the amino-terminus. We have started to use myc tagged Grb7 because it allows us to immunoprecipitate Grb7 with a second antibody. This will help detect Grb7 associated proteins (see last year's report and below). The second construct also had a myc tag but in addition had a CAAX box to localize Grb7 to the plasma membrane (15). The CAAX box would mimic the effect of Grb7 binding to growth factor receptors by localizing Grb7 to the plasma membrane. In this regard Grb7 may be similar to Grb2 which moves to the plasma membrane after binding growth factor receptors. Grb2 normally exists in the cytosol bound to the Ras activating protein, Son of sevenless (16). The



binding of Grb2 to the activated receptor results in the translocation of Grb2 and Son of sevenless to the membrane where Son of sevenless activates Ras. We hypothesize that Grb7 is also bound to other proteins and the movement of Grb7 to the membrane serves to activate signaling pathways. Thus by placing a CAAX box on Grb7 we felt we could permanently localize the protein to the plasma membrane and generate an activated form of Grb7. The placement of a CAAX box has resulted in the activation of several other signaling proteins (15,17).

CAAX-Grb7 generation was initiated by PCR of the CAAX box and polybasic region from the carboxy-terminus of H-Ras as well as PCR of the carboxy-region of Grb7. The primers were designed to allow the PCR product from H-Ras to anneal with the PCR product from Grb7. This allowed the generation of a construct containing the carboxy-terminus of Grb7 fused to the H-Ras CAAX box in a subsequent PCR. This fragment was then cloned into the mammalian expression vector, PMJ-30, using BstEII and BamHI sites. Both the myc tagged Grb7 and the CAAX box Grb7 were transfected into MDCK cells with a neomycin resistance plasmid using polybrene as previously described (18). Cell lines were obtained that contained myc-tagged Grb7 and CAAX-Grb7. Cells were lysed in hypotonic solution and the membrane fraction (P100) was separated from the cytosol (S100) by spinning at 100,000 x g. As expected all of the CAAX-Grb7 was found in the P100 membrane fraction (Fig. 1). It is interesting that in MDCK cells at least some of the myc-Grb7 is also at the membrane although this was not seen in every experiment.

With these cell lines we have evaluated the effects of Grb7 and CAAX-Grb7 overexpression on behavior of MDCK cells. As was found with MCF-7 cells, neither Grb7 nor CAAX-Grb7 had an effect on cell growth. The cells had a normal epithelial appearance and no gross differences in transport ability could be detected as assessed by dome formation (transport of fluid under the cells causing them to form domes). We next analyzed the cells for scattering ability (migration) in response to HGF. Cells were plated at 25,000 cells per well of a six well plate and stimulated with 50 units/ml of HGF for 18 hours. We found no effect of Grb7 overexpression on the ability of cells to scatter in response to HGF. This strongly suggests that Grb7 does not have a direct effect on cell migration. We are in the process of completing the tubulogenesis assay (13) in MDCK cells with Grb7 and CAAX-Grb7.

## **2) Protein interactions with Grb7.**

**i) Binding of G proteins to Grb7.** As outlined in Specific Aim #3 (Original Statement of Work), we have attempted to identify proteins that bind to the GM domain (central region) of Grb7 and Grb10. This year revealed a potential clue as to the role of the GM domain. A recently published sequence alignment suggested that Grb7 might have some similarity to proteins that interact with Ras (19). The region of similarity coincided exactly with the one hundred amino acids of the GM domain amino-terminal to the PH domain that we had previously identified (9). Ras is a small G-protein that is involved in many signaling pathways including the activation of the protein kinase Raf, the activation of other G-proteins such as Ral and possibly modulation of Phosphatidylinositol-3 Kinase activity (20). The mechanism by which Ras

controls these activities is by interacting with other signaling proteins in the GTP but not GDP bound state. The best understood is the interaction of GTP bound Ras with the amino-terminus of Raf (21). This serves to bring Raf to the plasma membrane where Raf is activated. Ras also binds to Ral-GDS, a protein that activates the small G-protein Ral (22). Grb7, Grb10 and Grb14 all have similarity to the Ras binding motif in Ral GDS and another Ras binding protein known as Canoe (19). Accordingly we examined if Ras can bind to the GM domain of Grb7. We expressed Ras in bacteria as a Glutathione-S-Transferase (GST) fusion protein and purified the protein on glutathione agarose. We then released Ras from the GST fusion using thrombin. The cleaved Ras was loaded with GDP or GTP by incubating the G protein in EDTA and then loading either guanyl nucleotide in the presence of a high concentration of magnesium. We then added GST-Raf or GST-Grb7 on beads to GTP or GDP loaded Ras and looked for precipitation of Ras by Raf or Grb7. We detected binding of GTP but not GDP Ras to Raf as has been previously published by several groups but disappointingly we found no binding of Grb7 to Ras (Figure 2). We are in the process of examining the binding of other small G proteins to Grb7. This has required us to generate the G-proteins Rho, Rac and CDC42 as GST fusion proteins. This has been completed and the binding experiments are presently being performed.

**ii) Binding of other proteins to Grb7.** We have not yet completed the studies measuring binding of Grb7 to radiolabelled proteins from cell lysates (see last year's report). Last year we proposed to examine proteins that coimmunoprecipitate with Grb7. To accomplish this we had planned to generate monoclonal antibodies against Grb7. We injected mice with a GST-Grb7 construct containing the SH2 and GM domains. We obtained mouse sera that reacted with Grb7 in ELISA assays at 1:1000 dilution. Although this was a relatively low titer we fused cells from one of these mice to myeloma cells to generate hybridomas. After screening over 300 fusions we did not obtain a useful monoclonal antibody. Part of the problem may have been that we were injecting mice with murine Grb7 and thus the mice did not generate a good immune response to the native antigen. We have decided not to pursue this monoclonal project further (i.e. clone the human Grb7 and use it as an immunogen) because we have generated several cell lines that contain Grb7 with a myc epitope (see above). The myc epitope will allow us to immunoprecipitate Grb7 with the anti-myc monoclonal antibody, 9E10, obviating the need for a Grb7 monoclonal antibody.

**3) Grb7 localization and its role in development.** Grb7 was initially cloned from an embryo library and found to be highly expressed in the liver and kidney of a six week mouse (3). We decided to further explore the expression of Grb7 in these two situations to begin to better understand the role of Grb7 in normal biology. We were able to determine the localization of Grb7 in the adult and embryonic kidney using RT-PCR. These studies were performed in collaboration with Dr. Josie Brigg's laboratory (Division of Nephrology, University of Michigan) who have prepared cDNA from different regions of adult rat kidney (23). First we needed to ensure that primers from the mouse Grb7 could amplify Rat cDNA by PCR. This was found to be the case and we proceeded to measure Grb7



expression in the different tubular segments of rat kidney. We found that Grb7 was expressed in all renal tubules suggesting a basic role of Grb7 in epithelial biology of the kidney (Fig. 3). We have also performed in situ hybridization of day 14 mouse embryo looking for Grb7 expression (collaboration with Dr. Greg Dressler, Dept. of Pathology, University of Michigan). Here we generated Grb7 probes using  $^{33}\text{P}$ -dUTP and T3 and T7 polymerase to generate sense and anti-sense Grb7 RNA probes. This was hybridized to mouse embryo using techniques previously developed by Dr. Dressler and the embryos examined by autoradiography (24). In the developing kidney we see early expression of Grb7 in cells as they make the transition from mesenchymal cells to epithelial cells again suggesting an important role for Grb7 in the basic function and formation of epithelial cells. We also detected large levels of Grb7 expression in lung and liver epithelia. Our plan is to complete our in situ analysis of Grb7 in developing organs using both earlier and later stage mouse embryos. These studies will lay the groundwork to perform similar studies in different stages of breast development.

## CONCLUSIONS

**1) Summary.** The results from our first two years of work can be summarized as follows:

i) We have transfected Grb7 into both transformed and nontransformed epithelial cells as initially proposed under Specific Aim #1 in the Statement of Work. Our studies have focused on cell growth and migration and we are now beginning to examine tubulogenesis of epithelial cells. Unfortunately we have not been able to detect a distinct phenotype in the Grb7 overexpressing cells. One of the major problems may be that all the cells we have worked with have a small amount of endogenous Grb7 and this may be sufficient for cellular signalling.

ii) We have searched for a binding partner for the GM domain of Grb7 as originally outlined in Specific Aim #3 in the Statement of Work. In the past year it has become clear that while the PH domain binds membrane lipids, the region just amino-terminal to the PH domain might be involved in binding small G-proteins or related molecules. We believe that proteins bind to this region of Grb7 although our initial approaches using expression cloning and GST pull down assays have been unsuccessful.

iii) We have begun examining the expression of Grb7 in normal mouse tissues where it appears to be localized to epithelial cells.

iv) Specific Aim #2 and Specific Aim #4 in the original Statement of Work have not been addressed to date. The reasons for this were outlined in last year's report.

**2) Future Plans.** Our future plans for this project are as follows:

i) Our inability to detect phenotypes in cells overexpressing Grb7 will necessitate a change in direction for the project. Because most epithelial cells contain a small amount of Grb7 we may continually have difficulty determining

the function of this protein by overexpression. Accordingly we have decided to delete Grb7 from the mouse genome (gene knockout) to determine its role in normal biology. If mice without Grb7 are nonviable it may give us a good idea as to the normal function of Grb7. If they survive they can be tested for the susceptibility to breast cancer induced by HER2. To accomplish these tasks we must first isolate clones containing the genomic sequence of Grb7. Using this genomic sequence we can generate a targeting vector to delete Grb7 from mouse embryonic ES cells. We will then inject these cells into mice to generate animals that are heterozygous and eventually homozygous for the Grb7 deletion. This work will be begun in the next year with the help of the transgenic core at The University of Michigan.

ii) We will continue to search for Grb7 binding partners. One approach will be to test several more small G-proteins for their binding to the Grb7 GM domain. Second we will search for proteins that are bound to Grb7 in the MDCK cells using anti-Grb7 and anti-myc immunoprecipitations on radioactively labelled cell lysates.

iii) We will complete our studies on Grb7 expression in mice using in situ hybridization. These results will be important in our future analysis of the Grb7 knockout mice.

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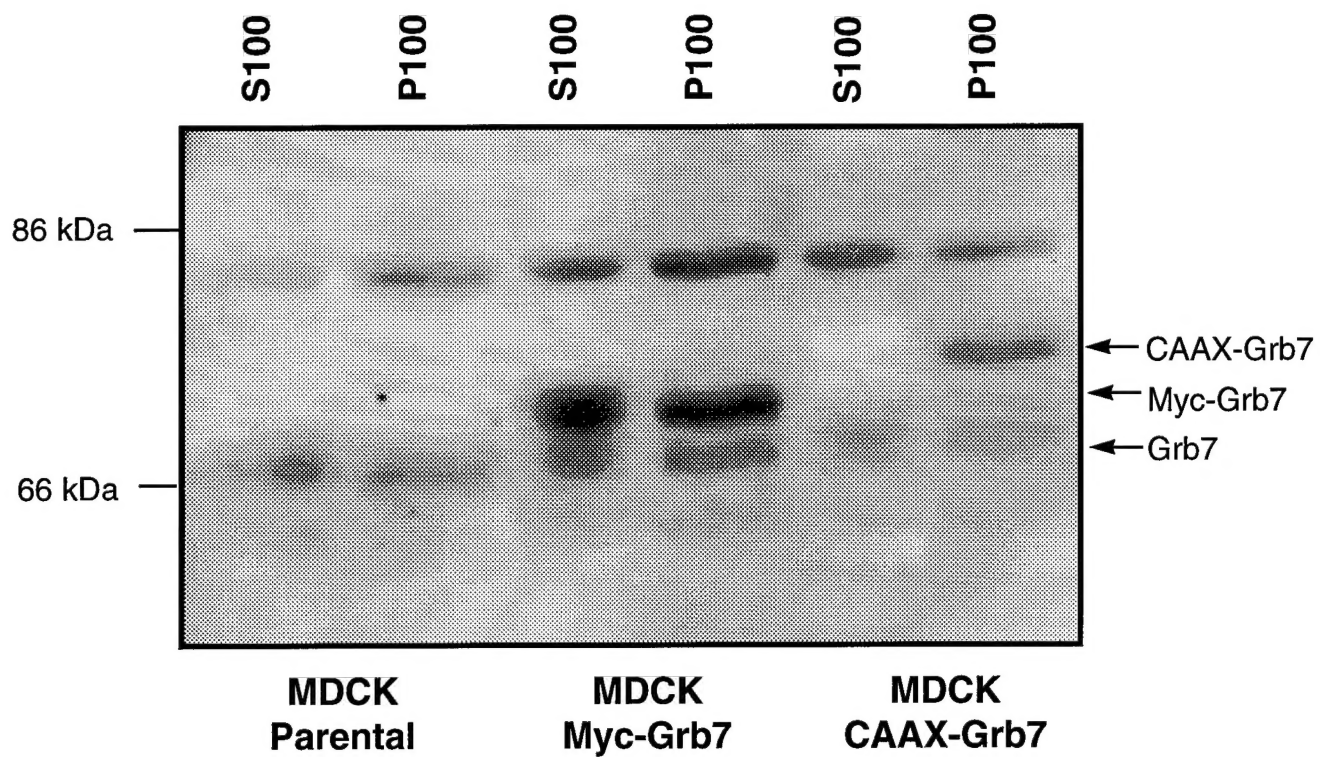
## APPENDIX

### FIGURES and LEGENDS

**Figure 1 Expression of Myc-Grb7 and CAAX-Grb7 in MDCK cells.** MDCK cells were transfected with Myc-Grb7 or CAAX-Grb7 in combination with a neomycin resistance plasmid. Clones were selected under G418 and tested for Grb7 expression. Parental MDCK cells and clones expressing Myc-Grb7 or CAAX Grb7 were then grown to confluence and lysed by douncing in hypotonic lysis buffer without detergent. A low speed spin was used to remove unlysed cells and nuclei and the resulting supernatant was spun at 100,000xg in an ultracentrifuge. The supernatant (S100) the pellet (P100) from this spin, were separated on an SDS-gel, transferred to nitrocellulose and blotted with anti-Grb7 antibodies. The P100 represents the membrane fraction and S100 the cytosolic fraction. Three times as much membrane were loaded in this experiment so the amount of Grb7 in the membrane is overestimated in this figure. Nonetheless it is clear that all the CAAX Grb7 is in the membrane. The various forms of Grb7 are indicated on the right of the panel. Myc Grb-7 runs larger than Grb7 due to the addition of the myc epitope and CAAX-Grb7 is even larger due to the further addition of the H-Ras CAAX box and polybasic region.

**Figure 2 Lack of binding of Ras to Grb7.** As discussed in the body of the text, a region in the Grb7 GM domain has similarities to domains that bind the small G-protein Ras. To test this, bacterially expressed Ras was loaded with GTP or GDP. The GTP or GDP Ras was added to GST, GST-Grb7 (full length), GST-Grb7-GM domain or GST-Raf. After binding for 90 minutes in the cold room, the samples were washed, run on a 15% SDS-gel, transferred to nitrocellulose and blotted with monoclonal anti-Ras antibodies. GST-Raf acted as a positive control and as expected bound GTP but not GDP Ras. There was however no binding to either of the Grb7 constructs or GST alone. The band at the top of the blot in each lane is a protein that nonspecifically interacts with the Ras antibody.

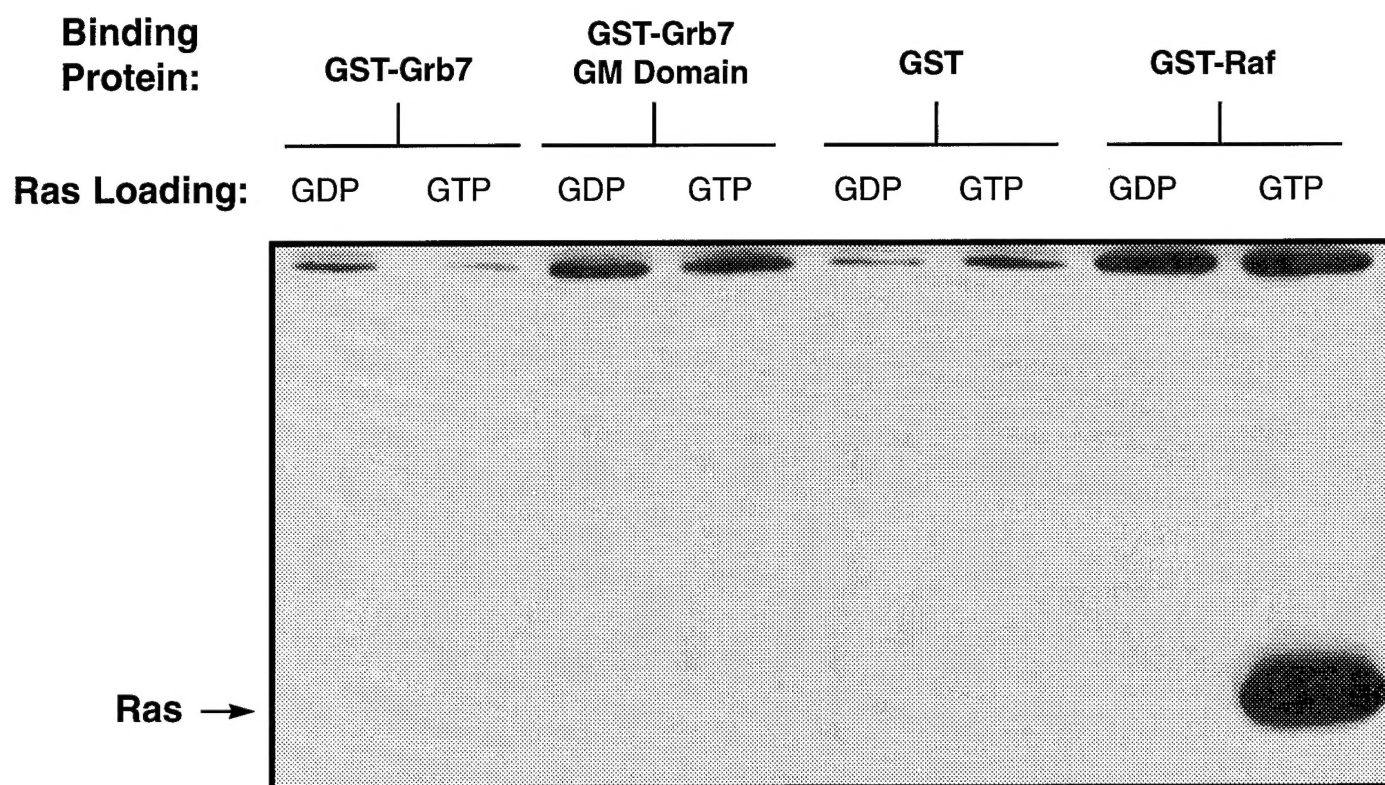
**Figure 3 Grb7 is present in all kidney tubular epithelia.** Individual nephron segments of rat kidney were dissected and first strand cDNA prepared as previously described (23). The cDNA was then amplified by PCR using primers from the SH2 domain of mouse Grb7. The reactions were performed using 34 cycles and a 54°C annealing temperature. The reactions contained 10 µCi of <sup>32</sup>P-dCTP to allow visualization of the PCR products. The band of 310 bp is the expected size for the Rat Grb7 PCR product. The results shown were obtained after 2 hours exposure using a phosphoimager. All tubular segments express Grb7. Arc. Art.-Arcuate Artery; Glom-Glomerulus; PCT-Proximal Convolved Tubule; PST-Proximal Straight Tubule; TDL-Thin Descending Limb; MTAL-Medullary Thick Ascending Limb; CTAL-Cortical Thick Ascending Limb; CCD-Cortical Collecting Duct; OMCD-Outer Medullary Collecting Duct; IMCD-Inner Medullary Collecting Duct.



**Blot: Anti-Grb7**

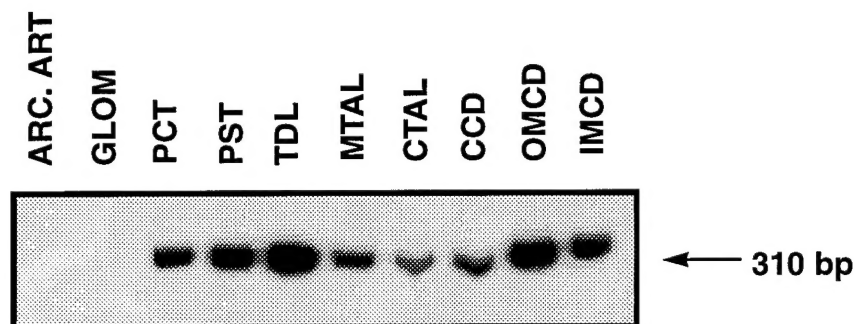
**FIG. 1**





**Blot: Anti-Ras**

**FIG. 2**



**FIG. 3**